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(54) Title: MULTIPLE-GENE MUTANTS OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) FOR VACCINE USE

(57) Abstract

The invention disclosed includes a method for the production of attenuated human immunodeficiency viruses (HIV). This method includes the production of a plasmid having a proviral HIV genome including the env, nef, vif, and vpr genes, and deleting from the plasmid significant portions of at least three, and preferably all four genes, such that the resulting plasmid encodes an attenuated virus that exhibits cell-free infectivity and reduced syncytium formation ability. Also disclosed is a method for the prophylactic prevention of infection of a person by HIV which comprises the administration of a prophylactically effective amount of attenuated virus, a vaccine for such prophylactic prevention, a method of therapeutic treatment comprising the administration of a therapeutically effective amount of attenuated virus and a vaccine for therapeutic treatment.

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MULTIPLE-GENE MUTANTS OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) FOR VACCINE USE

BACKGROUND OF THE INVENTION

Field of the Invention:

The invention herein relates to live-virus vaccines and their production and use.

Description of the Prior Art:

Live virus vaccines possess many desirable properties. Perhaps the most successful vaccines of any kind are those which employ live attenuated virus to elicit effective and extremely long lasting immunity in a very high proportion of immunized subjects. Live virus vaccines often induce effective immunity with a single immunization, making them ideal vaccines for large-scale use in developing countries, While killed virus vaccines can be effective, even against lethal agents, immunity is often short lived, such that multiple immunizations can be required even for transient protection.

Initially, killed virus vaccines against simian immunodeficiency virus (SIV) appeared to be quite effective against both homologous and heterologous strains, giving SIV impetus to the development of killed virus and subunit vaccines for human use. Unfortunately, most of the protection observed in earlier trials has been found to be due to protection elicited by human tissue antigens present in vaccine (and challenge) preparations. Animals have even been protectively immunized by preparations from uninfected human cells. Mutation of SIVmac239, with introduction of a deletion in the *nef* gene, has been reported to attenuate SIV virulence, and to provide protection from challenge with wild-type SIV strains. No similar reports have been made for either human immunodeficiency virus HIV-2 or HIV-1, and it is not clear that the situation with SIV can be reproduced with these viruses. In addition, it is

highly unlikely that a virus which persistently replicates and mutates in the host (as is the case for the SIV mutant) would be acceptable as a human vaccine except as a last resort under desperate circumstances.

Chimpanzees have been protected from HIV-1 by the use of killed virus preparations boosted by protein or peptide immunogens and/or by the use of envelope expressing vaccinia constructs, as well as by passively administered neutralizing antibody. The duration and scope of such protection is still under investigation, but preliminary reports indicate that protection may fail for divergent strains, or even the vaccine strain, as little as a year after an extensive vaccination series.

A more immediate application of attenuated virus vaccines may include their use as immunotherapeutic agents in the treatment of HIV infected individuals. At present, a number of therapeutic vaccines are in clinical trial, including the use of inactivated envelope-deficient virus and the use of recombinant envelope protein. In addition retrovirus vectored expression of HIV envelope protein is being developed as a therapeutic vaccine. The persistence, potency, and breadth of the immune responses elicited by these vaccines would be limited compared to that expected for a live attenuated virus vaccine.

SUMMARY OF THE INVENTION

The invention herein of safe and immunogenic molecular clones (mutants) of HIV-1, which have been altered to exhibit minimal cytopathogenicity, impaired and/or limited infectivity, and susceptibility to complete eradication by various non-toxic agents. These mutants of HIV-1 possess multiple genetic defects, including defects in the *vif*, *vpu*, *env*, *vpr*, and *nef* genes. Cell lines for production of several of these viruses (Molt-3/X295ΔS135, Molt3/X295ΔS) have also been double-cloned by limiting dilution and repeated screening.

A variety of mutations which had been previously examined for their syncytia forming potential, infectivity, and cytopathogenicity were used as starting material to create mutants of HIV-1 which were defective in multiple genes and/or gene regions. Starting clones include HXB2gpt (phenotypically *vpr*-, *vpu*-, *nef*-), HXB2ΔS (containing a large deletion in *vif* and *vpr*), X295 (containing a deletion of the carboxy-terminal envelope and *nef* regions in the HXB2 background), HXB2ala313 (constructed from HXB2, containing an envelope cassette from BH10 with a two-base mutation changing the "gpgr" motif at the tip of the V3 loop in *env* to "gagr"), and HXB2Δ135 containing a 84 bp deletion of the V3 loop in *env*, again in a HXB2 background containing the BH10 envelope cassette). Detailed descriptions of the starting materials and their methods of preparation will be found in Fisher et al, *Nature*, 316:262-265 (1985); Lee et al, *AIDS Res. and Human Retrovir.*, 5:441-449 (1989);and Ivanoff et al, *AIDS Res. and Human Retrovir.*, 7:595-603 (1991). Figure 2 illustrates the deletions of the basic types of mutant viruses created.

In its various aspects, the invention herein includes a method for the production of attenuated human immunodeficiency viruses (HIV) which comprises providing a plasmid comprising a proviral HIV clone and *env*, *nef*, *vif*, and *vpr* regions, and deleting from the plasmid significant portions of at least three of the *env*, *nef*, *vif*, and *vpr* regions, whereby the plasmid following deletion exhibits cell-free infectivity and reduced syncytium formation ability. Preferably deletions are made in all four of said regions. Also preferably the initial plasmid is pHXB2gpt or a mutant thereof having deletions in not more than two of the *env*, *nef*, *vif*, and *vpr* regions. Optionally the plasmid will also contain an *int* or *tat* region with a deletion therein.

Also part of the invention is a plasmid comprising a proviral HIV clone and *env*, *nef*, *vif*, and *vpr* regions, from which significant portions of initial lengths of at least three of the *env*, *nef*, *vif*, and *vpr* regions have been deleted, the resultant product plasmid with the deletions exhibiting cell-free infectivity and reduced syncytium formation ability. As with the method above, preferably deletions are made in all four of said regions. Also preferably the initial plasmid is pHXB2gpt or a mutant thereof having deletions in not more than two of the *env*, *nef*, *vif*, and *vpr* regions. Optionally the plasmid will also contain an *int* or *tat* region with a deletion therein. One may also include a polylinker for subcloning of at least one suicide gene.

Further as part of the invention is an attenuated human immunodeficiency virus comprising a plasmid comprising a proviral HIV clone and *env*, *nef*, *vif*, and *vpr* regions, from which significant portions of initial lengths of at least three of the *env*, *nef*, *vif*, and *vpr* regions have been deleted, the resulting plasmid with said deletions exhibiting cell-free infectivity and reduced syncytium formation ability. Again, preferably deletions are made in all four of said regions. Also preferably the initial plasmid is pHXB2gpt or a mutant thereof having deletions in not more than two of the *env*, *nef*, *vif*, and *vpr* regions. Optionally the plasmid will also contain an *int* or *tat* region with a deletion therein.

The invention also includes a method for prophylactic prevention of infection of a person by a human immunodeficiency virus which comprises administering to the person a prophylactically effective amount of the attenuated human immunodeficiency virus of this invention; a vaccine for such prophylactic prevention of infection; a method for therapeutic treatment of infection of a person infected by a human immunodeficiency virus which comprises administering to said person a therapeutically effective

amount of the attenuated human immunodeficiency virus of this invention; and a vaccine for therapeutic treatment (including amelioration or possible cure) of a person infected by a human immunodeficiency virus which comprises a therapeutically effective amount of the attenuated human immunodeficiency virus of this invention.

Vectored delivery is contemplated by the plasmid of this invention having an envelope devoid of promoter and polyadenylation signal regions; providing a method of vectored prophylactic prevention of infection of a person by a human immunodeficiency virus which comprises administering to said person a prophylactically effective amount of that plasmid encompassed in a replication-deficient virus; and providing a method of vectored therapeutic treatment of a person infected by a human immunodeficiency virus which comprises administering to said person a therapeutically effective amount of that plasmid encompassed in a replication-deficient virus.

While there is understandable hesitation when the feasibility of human live virus vaccines for retroviruses is under consideration, especially given the absence of good animal model for HIV-1 disease pathogenesis, the clones which we describe here address many of the safety concerns with respect to HIV live-virus vaccine candidates, and work in progress should provide a strategy providing sufficient reassurance to reliably lead to human trials of such an agent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of one of the starting materials, the HXB2gpt proviral plasmid, showing the location of various restriction sites.

Figure 2 is a schematic diagram of various multiple gene mutants of the present invention, showing the various regions and indicating where deletions occur.

Figure 3 is a graph showing reduced syncytia production by attenuated mutants adjusted for efficiency of

transfection [1 μg proviral DNA, HT4-LacZ (Magi-Emmerman) cells].

Figure 4 is a graph comparable *gag* p24 production by attenuated mutants adjusted for efficiency of transfection.

Figure 5 is a graph showing reduced killing by the ala313 mutant as compared to that of HXB2 (both at $100xTCID_{50}$], and including infectivity, cytopathogenicity and syncytia production.

Figure 6 is a graph showing the restoration of cell-free infectivity in the X295ΔS-*vif* mutant, with self-reinfectivity of frech human leukocytes as (SFU/ml-HT48C, TCID-PBMC) per ~1000 pg p24.

DETAILED DESCRIPTION AND PREFERRED EMBODIMENTS

The invention herein encompasses several different forms of the HIV-1 virus mutants. There are attenuated live viruses, i.e., viruses impaired in infectivity, cytopathogenicity, or actual pathogenicity which are capable of indefinite replication in the host, at some level; attenuated live viruses with suicide genes, i.e., viruses expressing genes which are normally harmless to the host but which can trigger cell death in the presence of pharmacologic agents (e.g. thymidine kinase and ganciclovir) allowing the populations of infected cells in the immunized host to be eradicated; controlled attenuated viruses, i.e., totally replication defective viruses which require addition of essential viral proteins as exogenous agents (e.g. *TAT* protein) in order to replicate In the host; and vectored or complemented replication defective viruses, i.e., viruses which will undergo only a single round or two rounds of replication which result in production of infectious virions due to complementation in producer lines or initial target cells, but which subsequently produce only non-infectious virions.

It has been known that one can make deletions in the carboxy-terminus of the HIV-1 *env* gene or mutations of the

HIV-1 V3 loop (in *env*) for reduced cytopathogenicity and altered replication characteristics of HIV in different cell types. In the present invention we have taken as the starting materials certain mutations defined further in the Fisher et al., Lee et al. and Ivanoff et al. references cited *supra*). To provide additional attenuation, we combined these mutations together with deletions of the *vif* and *vpr* genes of HIV-1, and, in addition, created similar clones with deletions in the 3'-LTR, which are incapable of producing infectious virus, for use in DNA virus vectored vaccines.

These mutations, which had been previously examined for their syncytia forming potential, infectivity, and cytopathogenicity, were used as starting material to create mutants of HIV-1 which were defective in multiple genes and/or gene regions. Specifically, the starting clones included HXB2gpt (phenotypically *vpr*-, *vpu*-, *nef*-), HXB2ΔS (containing a large deletion in *vif* and *vpr*, X295 (containing a deletion of the carboxy-terminal envelope and *nef* regions in the HXB2 background), HXB2ala313 (constructed from HXB2, containing an envelope cassette from BH10 with a two-base mutation changing the "gpgr" motif at the tip of the V3 loop to "gagr"), and HXB2Δ135 containing a 84 bp deletion of the V3 loop, again in a HXB2 background containing the BH10 envelope cassette). Mutant viruses created included HXB2ΔSala313, HXB2ΔSΔ135, X95ΔS, X295ala313, X295Δ135, X295ΔSala313, and X295ΔSΔ135 (some of which are shown in Figure 2).

All of these mutant viruses were found to be capable of producing p24 upon transfection, and of transactivating LTR-LacZ in HelaT4LacZ cells (see Figure 3). The syncytium forming capability of these clones (one measure of cytopathic affect) varied markedly, even when adjusted for p24 production and/or frequency of β-gal positive cells following transfection (see Figure 4) into HT4LacZ cells.

Previously, HXB2ala313 had been found to produce fewer and smaller syncytia (see Figure 5) as well as markedly reduced cell killing, even when adjusted for infectious dose. However, no reduction in syncytia formation had been observed with the ΔS or X295 strains, though X295 was noted to produce decreased cell killing in several *in vitro* systems. Unexpectedly, combining the X295 mutation with the ΔS mutation (X295ΔS -- a Δ*vif*, Δ*vpr*, *vpu*-, Δ*env*, Δ*nef* virus) resulted in substantial reduction in syncytium formation, while at the same time partially restoring cell-free infectivity to the *vif* deletion mutant (see Figure 6). All of the mutants containing either V3 loop mutation were essentially devoid of syncytium induction capability. Cell-free infectivity was reduced for many mutants, compared to wild-type (esp. for Δ135-containing clones, and uncompensated Δ*vif* mutants), but present for many others (such as X295ΔAS and X295ΔSala313). Most importantly, combination of the X295 *env-nef* deletion with the *vif-vpr* deletion significantly restored cell free infectivity, including cell-free infectivity for primary cells (peripheral blood mononuclear cells); see Figure 6.

Multiple producing clones have been derived (after transfection of Molt-3 cells) by double limiting-dilution cloning (second screening positive for p24 production) for X295ΔS, X295ΔSΔ135, and X295ΔSala313 clones (and others are in progress). These lines should prove extremely valuable in preparation of high-titer preparations for animal testing.

We used existing clones with deletions of COOH-*env*, *nef*, and the 3'-LTR (X194, X358) to move these deletions into previous created double- and triple-mutants clones above (taking advantage of the BamHI-FspI unique plasmid fragment containing these deletions) in the background of BH10 envelope derived clones (ala313 and Δ135), and reconstructed a BH10 envelope in the pHXB2D based ΔS mutant by moving a SaII-BamHI fragment of HX10 envelope into this

clone after replacement of the BamHI-FspI fragment. These plasmids provide a convenient SstI plasmid fragment, devoid of promoter and polyadenylation signal, which can be moved into other expression cassettes. When inserted into replication-deficient DNA viruses (CMV, HSV and BPV vectors) this is believed to be capable of providing all of the advantages of live-virus vaccines (complete particle production, intracellular production of proteins, persistent presentation for several weeks to months) in the context of a proviral clone which is totally incapable of producing infections particles due to the deletion in the 3'-LTR (required for successful reverse-transcription and integration.)

The invention also encompasses clones with deletions of additional genes, including clones with deletions in the first coding exon of *tat* and an *int* mutant which would be limited to a single round of infection, with subsequent production of intact yet non-infectious virus particles. These are constructed in the background of the X295ΔS and X295ΔSala313 clones. Such additional mutations may also be moved into the LTR-deleted X194 based clones. *TAT* defective mutants may be created by PCR mutagenesis, with introduction of three stop codons into the *tat* first exon, to complement with recombinant *TAT* protein. Creation of integrase mutants and complementary producing lines may be achieved by inserting multiple frame stop cloning sites at the beginning of *int* by PCR mutagenesis, and deleting *int* and remaining *vpr* (whole central region of the virus) to just before the *tat* splice acceptor (SaII site), while amplifying *int* with other primer pairs (with introduction of a consensus start signal, acylation signal, and protease cleavage signal) for cloning into a CMV promoter plasmid. Plasmids such as pMAMneo, pRc/RSV, pRe/CMV and pcDNAIneo each have some distinct advantages and liabilities, but those skilled in the art will be easily able to select

appropriate vectors in different situations for expression *in trans*, leaving no shared sequences with the proviral plasmid.

The *vif-vpr* deletion in native X295 may be reconstructed, using a unique XcmI-NcoI sites, to insert a polylinker for subcloning of one or two suicide genes,such as HSV *tk* and bacterial *xgpt* genes, into the viral *vif* and *nef* reading frames. Both of these genes allow both positive and negative selection using certain cell lines and agents *in vitro*, allowing easy selection of producing clones using mycophenolic acid/HAT selection, etc. Negative selection is included to permit *in vivo* eradication of expressing clones. In addition, while ganciclovir killing of expressing *tk* clones may be effective, the addition of a second gene increases the likelihood of complete eradication of cells infected with virus, as well as giving an alternative suicide agent (6-MP) which is not a frequently used and often desperately and needed drug, as are acyclovir and ganciclovir. We anticipate that this feature will be required for introduction into humans.

The SaII-BamHI unique sites have been preserved in each of these clones, and allow for facile insertion of envelope fragments from a variety of HIV-1 isolates. HIV-1_{MN} and wild-type strain envelopes may be amplified for this purpose. This should alleviate any expectation that since HXB2 (from III_B/LAV/LAI) is not a prevalent strain, it would not be particularly appropriate for vaccine purposes, especially with respect to envelope coding sequences. It is, however, not clear that the diversity of HIV-1 in *env* need be represented in a live virus vaccine, but this option is included since presence of such sites allows swapping of ~1.5 Kb envelope fragments amplified from wild-type strains using PCR into the SaII- BamHI envelope region of the attenuated clones.

Other producing cell lines may be needed, though the Molt-3 line is not virally infected, and the need for *vif* permissive line for production may not allow the use of nontransformed lines, such as MRC-5 and the like.

Characterization of clones *in vitro* is by a variety of means, including confirmation of expected patterns of protein expression; replication in primary cells, such as CD4+ lymphocytes and monocytes; replication in cell lines; syncytium forming capability *in vitro*; cell killing *in vitro*; and demonstration of eradication of clones containing suicide genes by selective agents *in vitro*. Selection and characterization of producing lines and virus preparations for inoculation must adhere to good manufacturing practices standard for animal and human administration.

The various mutants may be tested in animal models in a number of different types of experiments, including but not limited to establishment of infectivity in macaque cells; infectivity for *M. nomestrina*; exploration of cutaneous inoculation; evaluation of proviral persistence; evaluation of immunogenicity, including neutralizing antibody, ADCC, and cytolytic activity; and eradication of clones containing suicide genes.

The route of administration in humans is expected to be by cutaneous (intradermal) or subcutaneous administration. Both single and multiple injections are contemplated.

It will be evident to those skilled in the art that there are many other variations which, while not expressly set forth above, are clearly within the scope and spirit of the invention. The above description is therefore intended to be exemplary only, and the scope of the invention is to be determine solely from the appended claims.

WE CLAIM:

CLAIMS

1. A method for the production of attenuated human immunodeficiency viruses (HIV) which comprises:

providing a plasmid comprising a proviral HIV clone and *env*, *nef*, *vif*, and *vpr* regions; and

deleting from said plasmid significant portions of at least three of said *env*, *nef*, *vif*, and *vpr* regions;

whereby said plasmid following said deletion exhibits cell-free infectivity and reduced syncytium formation ability.

2. A method as in Claim 1 wherein said deletions are made in all four of said regions.

3. A method as in Claim 1 wherein said plasmid prior to said deletions is pHXB2gpt or a mutant thereof having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

4. A method as in Claim 2 wherein said plasmid prior to said deletions is pHXB2gpt or a mutant thereof having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

5. A method as in Claim 1 wherein said plasmid further comprises at least an *int* or *tat* region and a deletion is also made therein.

6. A plasmid comprising a proviral HIV clone and *env*, *nef*, *vif*, and *vpr* regions, from which significant portions of initial lengths of at least three of said *env*, *nef*, *vif*, and *vpr* regions have been deleted, said plasmid with said deletions exhibiting cell-free infectivity and reduced syncytium formation ability.

7. A plasmid as in Claim 6 wherein significant portions of said initial lengths of all four of said regions have been deleted.

8. A plasmid as in Claim 6 comprising before said deletions pHXB2gpt or a mutant thereof having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

9. A plasmid as in Claim 7 comprising before said deletions pHXB2gpt or a mutant thereof having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

10. A plasmid as in Claim 6 further comprising at least an *int* or *tat* region with a deletion from initial length also made therein.

11. An attenuated human immunodeficiency virus comprising a plasmid comprising a proviral HIV clone and *env*, *nef*, *vif*, and *vpr* regions, from which significant portions of initial lengths of at least three of said *env*, *nef*, *vif*, and *vpr* regions have been deleted, said plasmid with said deletions exhibiting cell-free infectivity and reduced syncytium formation ability.

12. An attenuated human immunodeficiency virus as in Claim 11 wherein significant portions of said initial lengths of all four of said regions have been deleted.

13. An attenuated human immunodeficiency virus as in Claim 11 wherein said plasmid before said deletions comprises pHXB2gpt or a mutant thereof having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

14. An attenuated human immunodeficiency virus as in Claim 12 wherein said plasmid before said deletions comprises pHXB2gpt or a mutant thereof having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

15. An attenuated human immunodeficiency virus as in Claim 11 wherein said plasmid further comprises at least an *int* or *tat* region with a deletion from initial length also made therein.

16. A method for prophylactic prevention of infection of a person by a human immunodeficiency virus which comprises administering to said person a prophylactically effective amount of an attenuated human immunodeficiency virus as in any of Claims 11, 12, 13, 14 or 15.

17. A vaccine for prophylactic prevention of infection of a person by a human immunodeficiency virus which comprises a prophylactically effective amount of an attenuated human immunodeficiency virus as in any of Claims 11, 12, 13, 14 or 15 in a suitable administrable carrier.

18. A method for therapeutic treatment of infection of a person infected by a human immunodeficiency virus which comprises administering to said person a therapeutically effective amount of an attenuated human immunodeficiency virus as in any of Claims 11, 12, 13, 14 or 15.

19. A vaccine for therapeutic treatment of a person infected by a human immunodeficiency virus which comprises a therapeutically effective amount of an attenuated human immunodeficiency virus as in any of Claims 11, 12, 13, 14 or 15 in a suitable administrable carrier.

20. A plasmid as in any one of Claims 6, 7, 8, 9 or 10 further comprising at least one of an envelope devoid of promoter and polyadenylation signal regions and a polylinker for subcloning of at least one suicide gene.

21. A method of vectored prophylactic prevention of infection of a person by a human immunodeficiency virus which comprises administering to said person a prophylactically effective amount of a plasmid as in Claim 20 encompassed in a replication-deficient virus.

22. A method of vectored therapeutic treatment of a person infected by a human immunodeficiency virus which comprises administering to said person a therapeutically effective amount of a plasmid as in Claim 20 encompassed in a replication-deficient virus.

AMENDED CLAIMS

[received by the International Bureau on 27 May 1994 (27.05.94); original claims 3,4,6-15 amended; remaining claims unchanged (4 pages)]

1. A method for the production of attenuated human immunodeficiency viruses (HIV) which comprises:

providing a plasmid comprising a proviral HIV clone and *env*, *nef*, *vif*, and *vpr* regions; and

deleting from said plasmid significant portions of at least three of said *env*, *nef*, *vif*, and *vpr* regions;

whereby said plasmid following said deletion exhibits cell-free infectivity and reduced syncytium formation ability.

2. A method as in Claim 1 wherein said deletions are made in all four of said regions.

3. A method as in Claim 1 wherein said plasmid prior to said deletions is pHXB2gpt having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

4. A method as in Claim 2 wherein said plasmid prior to said deletions is pHXB2gpt having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

5. A method as in Claim 1 wherein said plasmid further comprises at least an *int* or *tat* region and a deletion is also made therein.

6. A plasmid comprising a proviral HIV clone and *env*, *nef*, *vif*, and *vpr* regions, from which significant portions of at least three of said *env*, *nef*, *vif*, and *vpr* regions have been deleted, said plasmid with said deletions exhibiting cell-free infectivity and reduced syncytium formation ability.

AMENDED SHEET (ARTICLE 19)

7. A plasmid as in Claim 6 wherein significant portions of all four of said regions have been deleted.

8. A plasmid as in Claim 6 comprising before said deletions pHXB2gpt having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

9. A plasmid as in Claim 7 comprising before said deletions pHXB2gpt having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

10. A plasmid as in Claim 6 further comprising at least an *int* or *tat* region with a deletion also made therein.

11. An attenuated human immunodeficiency virus comprising a plasmid comprising a proviral HIV clone and *env*, *nef*, *vif*, and *vpr* regions, from which significant portions of at least three of said *env*, *nef*, *vif*, and *vpr* regions have been deleted, said plasmid with said deletions exhibiting cell-free infectivity and reduced syncytium formation ability.

12. An attenuated human immunodeficiency virus as in Claim 11 wherein significant portions of all four of said regions have been deleted.

13. An attenuated human immunodeficiency virus as in Claim 11 wherein said plasmid before said deletions comprises pHXB2gpt having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

14. An attenuated human immunodeficiency virus as in Claim 12 wherein said plasmid before said deletions comprises pHXB2gpt having deletions in not more than two of said *env*, *nef*, *vif*, and *vpr* regions.

15. An attenuated human immunodeficiency virus as in Claim 11 wherein said plasmid further comprises at least an *int* or *tat* region with a deletion also made therein.

16. A method for prophylactic prevention of infection of a person by a human immunodeficiency virus which comprises administering to said person a prophylactically effective amount of an attenuated human immunodeficiency virus as in any of Claims 11, 12, 13, 14 or 15.

17. A vaccine for prophylactic prevention of infection of a person by a human immunodeficiency virus which comprises a prophylactically effective amount of an attenuated human immunodeficiency virus as in any of Claims 11, 12, 13, 14 or 15 in a suitable administrable carrier.

18. A method for therapeutic treatment of infection of a person infected by a human immunodeficiency virus which comprises administering to said person a therapeutically effective amount of an attenuated human immunodeficiency virus as in any of Claims 11, 12, 13, 14 or 15.

19. A vaccine for therapeutic treatment of a person infected by a human immunodeficiency virus which comprises a therapeutically effective amount of an attenuated human immunodeficiency virus as in any of Claims 11, 12, 13, 14 or 15 in a suitable administrable carrier.

20. A plasmid as in any one of Claims 6, 7, 8, 9 or 10 further comprising at least one of an envelope devoid of promoter and polyadenylation signal regions and a polylinker for subcloning of at least one suicide gene.

AMENDED SHEET (ARTICLE 19)

21. A method of vectored prophylactic prevention of infection of a person by a human immunodeficiency virus which comprises administering to said person a prophylactically effective amount of a plasmid as in Claim 20 encompassed in a replication-deficient virus.

22. A method of vectored therapeutic treatment of a person infected by a human immunodeficiency virus which comprises administering to said person a therapeutically effective amount of a plasmid as in Claim 20 encompassed in a replication-deficient virus.

AMENDED SHEET (ARTICLE 19)

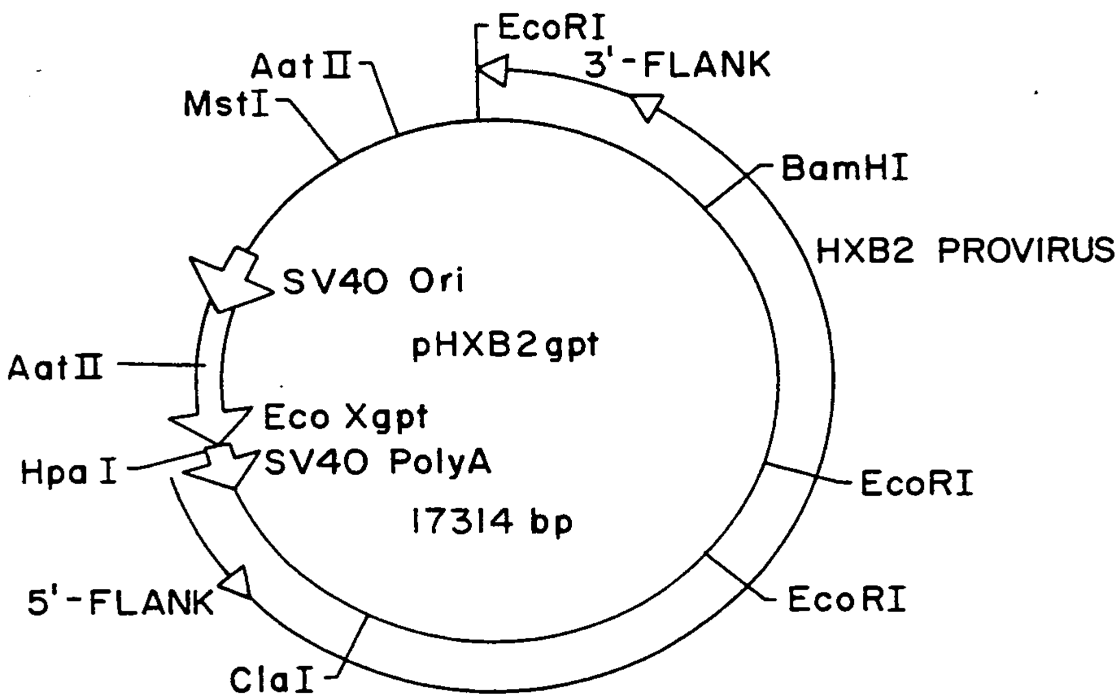


FIG. I

SUBSTITUTE SHEET (RULE 26)

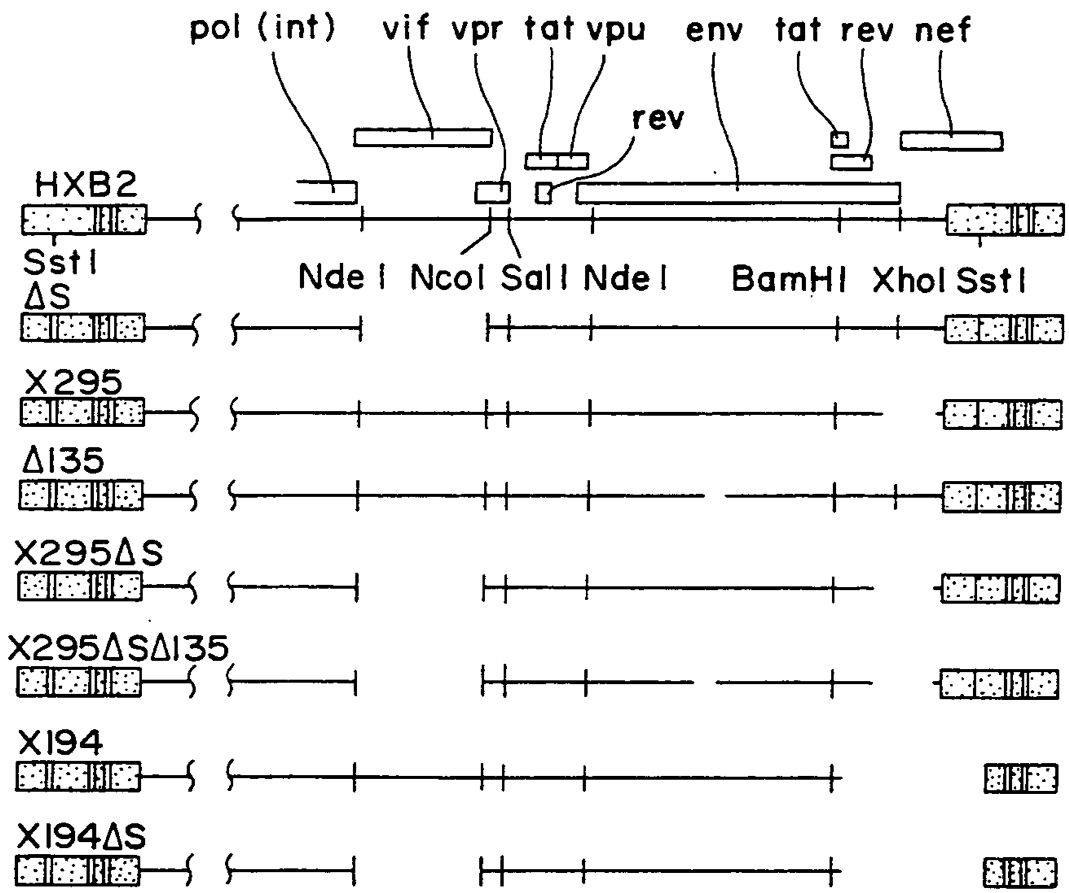


FIG. 2

SUBSTITUTE SHEET (RULE 26)

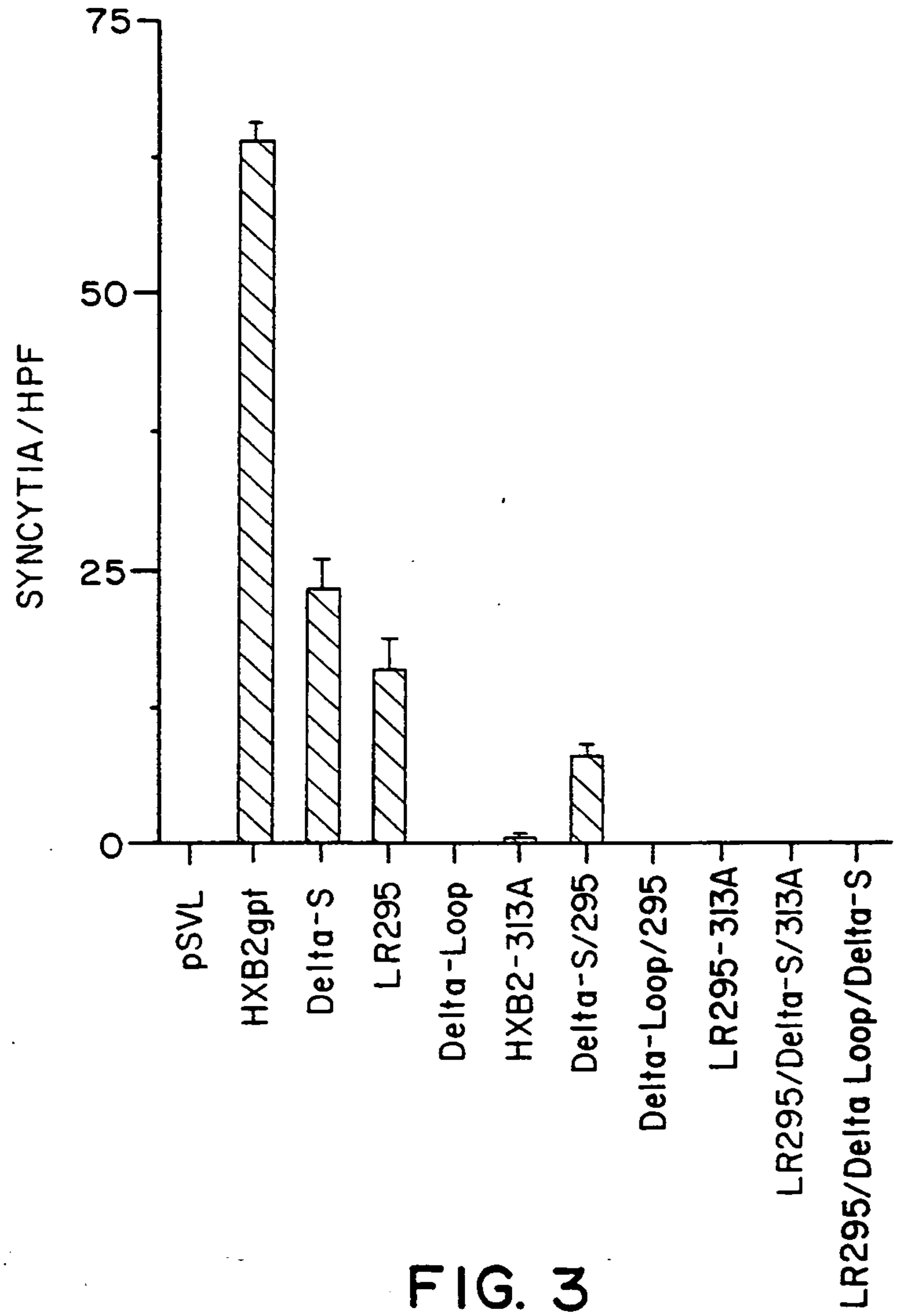

SYNCYTIA/HPF
75
50
25
0
pSVL
HXB2gpt
Delta-S
LR295
Delta-Loop
HXB2-313A
Delta-S/295
Delta-Loop/295
LR295-313A
LR295/Delta-S/313A
LR295/Delta Loop/Delta-S

FIG. 3

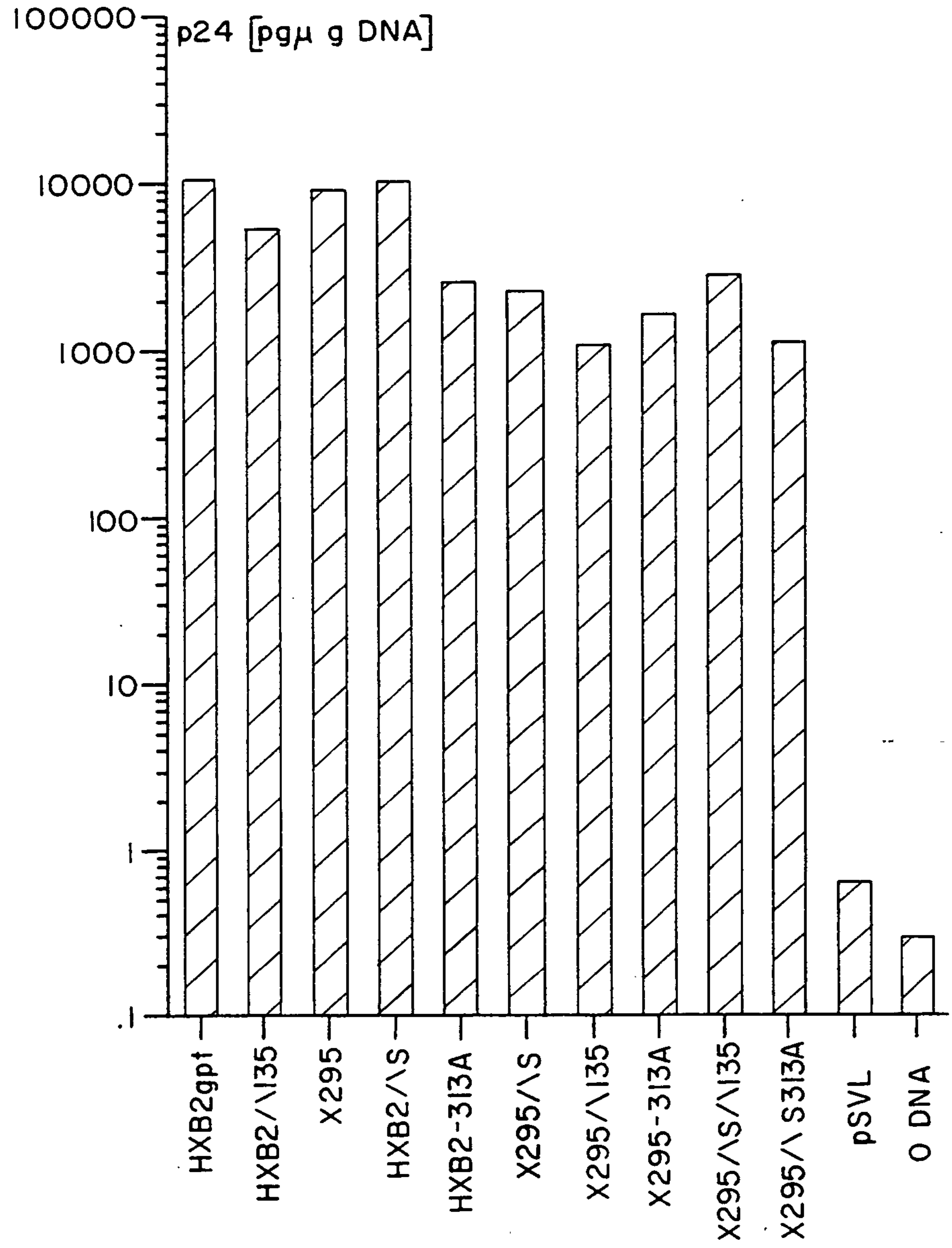

p24 [pgμ g DNA]
100000
10000
1000
100
10
1
.1
HXB2gpt
HXB2/\I35
X295
HXB2/\S
HXB2-313A
X295/\S
X295/\I35
X295-313A
X295/\S/\I35
X295/\ S313A
pSVL
O DNA

FIG. 4

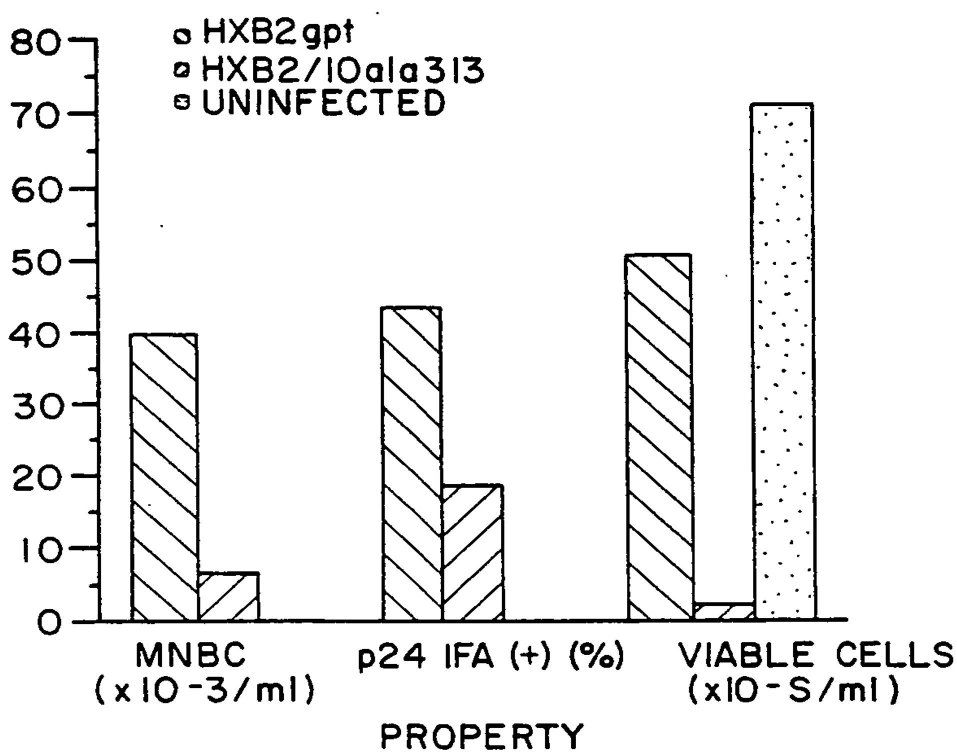
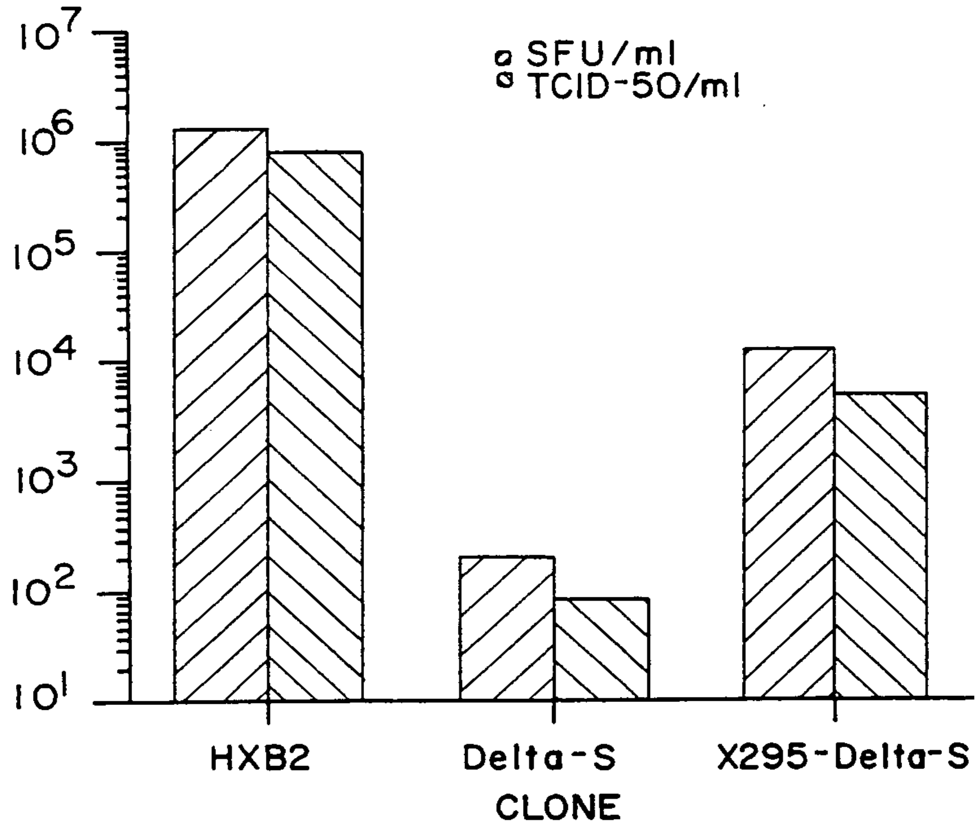

80
70
60
50
40
30
20
10
0
HXB2gpt
HXB2/10ala313
UNINFECTED
MNBC
(x10-3/ml)
p24 IFA (+) (%)
VIABLE CELLS
(x10-S/ml)
PROPERTY

FIG. 5



10^7
10^6
10^5
10^4
10^3
10^2
10^1
SFU/ml
TCID-50/ml
HXB2
Delta-S
X295-Delta-S
CLONE

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12088

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/12, 39/21; C12N 7/00, 7/04, 15/00, 15/33, 15/48
US CL :424/88, 89, 93A, 93T; 435/235.1, 236, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 89, 93A, 93T; 435/235.1, 236, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APA, MEDLINE, BIOSIS, EMBASE, DERWENT
seacrh terms: HIV, attenuate, avirulent, nonvirulent, deletion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Immunobiology, Volume 184, Number 2/3, issued February 1992, S. Norley et al., "Vaccination against HIV", pages 193-207, see entire document.	1-22
X -- Y	AIDS Research and Human Retroviruses, Volume 5, Number 4, issued 1989, S.J. Lee et al., "Role of the Carboxy-Terminal Portion of the HIV-1 Transmembrane Protein in Viral Transmission and Cytopathogenicity", pages 441-449, see entire document.	3-4,8-9,13-14 ---------------- 1-2,5-7,10-12,15-22
Y	WO, A, 92/00987 (DESROSIERS ET AL.) 23 January 1992, see entire document, especially page 19.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 17 March 1994	Date of mailing of the international search report MAR 29 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Michael S. Tuscan Ph.D. Jill Warden for
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

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BNSDOCID: <WO___9417825A1_I_>

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AIDS Research and Human Retroviruses, Volume 8, Number 8, issued August 1992, R.C. Desrosiers, "HIV with Multiple Gene Deletions as a Live Attenuated Vaccine for AIDS", page 1457, see entire document.	1-22
Y	AIDS Research and Human Retroviruses, Volume 8, Number 3, issued March 1992, R.C. Desrosiers, "HIV with Multiple Gene Deletions as a Live Attenuated Vaccine for AIDS", pages 411-421, see entire document, especially pages 14 and 15.	1-22
Y	Science, Volume 258, issued 18 December 1992, M.D. Daniel et al., "Protective Effects of a Live Attenuated SIV Vaccine with a Deletion in the nef Gene", pages 1938-1941, see entire document.	1-22
Y	Journal of Virology, Volume 65, Number 11, issued November 1991, D. Dedera et al., "Demonstration of Two Distinct Cytopathic Effects with Syncytium Formation-Defective Human Immunodeficiency Virus Type 1 Mutants", pages 6129-6136, see entire document, especially Figure 1.	1-22
Y	Proceedings of the National Academy of Sciences USA, Volume 87, issued October 1990, N. Hattori et al., "The Human Immunodeficiency Virus Type 2 vpr Gene is Essential for Productive Infection of Human Macrophages", pages 8080-8084, see entire document, especially Abstract and page 8083.	1-22
Y	The New Biologist, Volume 3, Number 7, issued July 1991, G. Plautz et al., "Selective Elimination of Recombinant Genes in Vivo with a Suicide Retroviral Vector", pages 709-715, see entire document.	1-22

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